MicroRNA-34a underexpressed in Merkel cell polyomavirus –negative Merkel cell carcinoma tumors

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Tiivistelmä – Referat – Abstract

Introduction:
Merkel cell carcinoma (MCC) is a rare neuroendocrine cancer of the skin that has a strong propensity to relapse and metastasize. Exposure to ultraviolet radiation and immunosuppression contribute to MCC. Merkel cell polyomavirus (MCV) is detected in 70% to 80% of MCC tumors, but the significance of MCV infection is not yet understood. MicroRNAs (miRNA) have been reported to associate with many types of cancer, and miRNA profiles of other cancers with a virus etiology have been defined. The aim of this study was to compare the expression of five miRNAs, miR-34a, miR-30a, miR-1539, miR-142-3p and miR-181d in formalin fixed paraffin embedded MCC tumor samples according to MCV status using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Materials and methods:
Sufficient RNA was extracted from 26 tumor samples and from control skin sample using the miRNeasy FFPE Kit (QIAGEN, Valencia, CA, USA). Reverse transcription of the RNA was done using the miScript II RT Kit (50) (QIAGEN). QRT-PCR was executed with miScript SYBR Green PCR Kit (QIAGEN).and LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany). Student's t-test and Mann-Whitney U-test were used to evaluate differences in miRNA expression.

Results:
We found a statistically significant underexpression of mir-34a in MCV-negative tumors compared to MCV-positives. The other four miRNAs studied did not show significant expression differences according to MCV-status. There was no statistically significant difference in miRNA expression according to tumor location or metastasizing.

Conclusion:
The difference in miRNA expression according to MCV-status suggests distinct pathogenesis of the tumors. The role of underexpressed miR-34a in MCV-negative tumor pathogenesis remains unclear but it might be consequential in the tumorigenesis of MCV-negative tumors.

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1 Introduction

1.1 Merkel cell carcinoma

Merkel cell carcinoma (MCC) is a rare neuroendocrine cancer of the skin that mainly affects the elderly population. It has a strong propensity to relapse and metastasize, both locally and systemically (1). The regional lymph nodes are a common site for metastasis. The cells of origin in MCC remain ambiguous (2).

The diagnosis is based on histology combined with immunohistochemical studies. Cytokeratin-20 should stain positively and the thyroid transcription factor-1 negatively (3-5). There are three histological subtypes: the trabecular subtype, the intermediate subtype and the small cell type. (6,7) The intermediate subtype is the most universal, and together with the small cell type, they are the aggressive forms of the disease.

1.2 Cells of origin

MCC has been thought to rise from Merkel cells, cells of the basal layer of epidermis. Merkel cells are most abundant in areas of special sensory function, fingertips for example (8). They constitute Merkel cell-axon complexes with the primary nerve endings of the skin (9). Merkel cells have been proposed to rise from the neural crest (10,11) but their origin as well as their function still remains controversial (12). Merkel cells have been the primary candidate for cells of origin in MCC due to the appearance of neuroendocrine granules and cytokeratine-20 in MCC tumor cells (13,14). But their post mitotic state (15) and location in the epidermis oppose the fact that MCC tumors primarily reside in the dermis, and proliferative activity is frequently observed in MCC tumor cells (16,17). Epidermal stem cells are prospective cells of origin, but experimental evidence is yet to be reported (2). The etiology of the MCC has not been entirely solved, but exposure to ultraviolet radiation (18-20) and immunosuppression contribute to MCC (21-24).

1.3 Prognosis and treatment

The five-year survival rate of MCC patients is around 70% (25-28), but involvement of the local lymph nodes and more distant metastases reduce the survival rates considerably (29,30). Sentinel lymph node biopsy (SLNB) has been shown to be an important tool to define the stage of MCC. A positive SLNB may predict the progression of local metastases (31). Tumor size < 2cm is a favorable prognostic factor (32). No treatment protocol has been accepted unanimously due to scarce patient material, but surgical excision and SNLB followed by radiation treatment to the local skin area is the common course of treatment (33).

1.4 Merkel cell polyomavirus

In 2008, a new polyomavirus was detected in 80% of a compilation of MCC samples (34). The virus was named Merkel cell polyomavirus (MCV) and has ever since been reported in 70% to 80% of the MCCs explored (35-39). Immunocompromised patients have an even higher incidence of MCV in their tumors (40,41). Virus positive patients have a more preferable course of the disease (39), yet there are studies contradicting this conclusion (42,43). Therefore the significance of the MCV infection is not yet completely perceived.

MCV DNA integrates clonally into the host cell genome (34) and undergoes truncating mutations in the MCV large T antigen (LT) helicase, which incapacitates the viral replication machinery (44). Li et al. showed that a truncating mutation of the MCV LT, which deletes the C-terminal p53 tumor suppressor activating region while retaining the N-terminal Retinoblastoma inhibiting domain, is likely to be a requirement for the infected cells to become tumorigenic (45). Clonal integration is evident in the primary tumors and in metastases as well, suggesting that the proliferation of tumor cells occur after the integration of the viral DNA (46,47).

1.5 MicroRNAs and cancer

MicroRNAs (miRNA) are small non-coding RNA molecules of about 20-25 nucleotides. MiRNAs are known to have an important role in controlling cellular development, differentiation, proliferation and apoptosis. They participate in cellular processes by negatively regulating gene expression at the transcriptional or post-transcriptional level (48,49). In addition to DNA mutation, epigenetic mechanisms like DNA methylation and Histone modification regulate miRNA gene expression (50). Particularly methylation of the genomic sequences called CpG islands firmly represses the transcription of those regions, and many miRNA genes relate to these CpG islands (50). MiRNAs have been reported to associate with many types of cancer (51,52), for example chronic lymphocytic leukaemia, glioblastoma and breast cancer as well as cervical cancer, but the key miRNAs in MCC pathogenesis remain unidentified. In 2011, Lee et al. reported that the MCV genome encodes a viral miRNA that is involved in the virus's escape from immunosurveillance (53).

MiRNA profiling for other cancers with viral etiology has been done previously at least for the human papillomavirus (HPV) associated cervical cancer and the Epstein-Barr virus-associated NK/T-cell lymphomas (54,55). In 2013 Xie et al. evaluated miRNA expression profiles in MCC tumors according to the MCV status and found distinct expression profiles for MCV positive and negative tumors (56). Our group, led by Sahi in 2013, used microarray techniques to compare microRNA expression between MCV positive and negative tumors. We found four miRNAs to be underexpressed in MCV negative tumors. These miRNAs were miR-34a, miR-30a, miR-1539 and miR-142-3p. The only miRNA overexpressed was miR-181d.

1.6 Aim of the study

The aim of this present study was to compare the expression of five miRNAs, miR-34a, miR-30a, miR-1539, miR-142-3p and miR-181d in MCC tumor samples according to MCV status, using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) and potentially validate these differences in miRNA regulation. The results were linked to patients' clinical parameters and analyzed statistically.

2 Materials and Methods

2.1 Tumor samples

Data on patients diagnosed with MCC in Finland during 1979-2004 was obtained from the Finnish Cancer Registry and Helsinki University Hospital files. Clinical details, such as age at diagnosis, gender, tumor size and location as well as data of other diseases and metastasis were extracted from hospital records. Formalin-fixed, paraffin-embedded tissue blocks were accordingly retrieved from the pathology archives. The study was approved by the local ethics committee.

The MCC diagnoses were confirmed in a blinded fashion during our earlier studies by two researchers with special expertise in MCC pathology (Tom Böhling and Heli Kukko). The samples were stained with hematoxylin and eosin, and we performed immunohistochemistry with antibodies for cytokeratin-20 (CK-20, DakoCytomation, Glostrup, Denmark) and thyroid transcription factor-1 (TTF-1; Novocastra, Balliol Business Park West, Benton Lane, Newcastle Upon Tyne, UK). We required for the histological diagnosis of MCC 1. that the morphology was compatible with MCC in light microscopy, 2. positive staining for CK-20 and 3. negative staining for TTF-1.

In our previous study, the presence of MCV DNA was analyzed from DNA extracted from representative deparaffinized tumor sections (39). Quantitation of MCV DNA was done using real-time PCR. The relative DNA sequence copy number for each tissue sample was expressed as a ratio of MCV DNA-to-protein tyrosine phosphatase gamma receptor gene (PTPRG) DNA. When the MCPyV DNA to *PTPRG* DNA ratio was >0 the sample was considered positive (39).

For this study, 14 MCV-positive and 13 MCV-negative MCC tumors and one control sample from healthy skin were chosen based on the amount of paraffinized tumor sample available.

2.2 RNA extraction

RNA was extracted from 27 tumor samples and from a control skin sample. Extraction was done using the miRNeasy FFPE Kit (QIAGEN, Valencia, CA, USA cat.no. 217504). For deparaffinization ,we used the Deparaffinization Solution (QIAGEN, cat.no. 19093).

Extraction steps were*:*

1. *160 µl Deparaffinization Solution was added to microcentrifuge tubes containing the tumor sample sections. Samples were vortexed for 10 seconds and centrifuged briefly.*
2. *Samples were incubated at 56°C for 3 min, then allowed to cool to room temperature.*
3. *150µl of Buffer PKD was added, and the samples were mixed by vortexing and then centrifuged for 1 min at 11,000 x g (10,000 rpm).*
4. *10µl of proteinase K was added to the lower, clear phase. Mixing was done gently by pipetting up and down.*
5. *Samples were incubated at 56°C for 15 min, then at 80°C for 15 min.*
6. *Lower, clear phase was transferred into a new microcentrifuge tube, and the samples were incubated on ice for 3 min. Then they were centrifuged for 15 min at 16,100 x g.*
7. *The supernatant was transferred to a new microcentrifuge tube without disturbing the pellet.*
8. *16µl DNAse Booster Buffer and 10µl DNase I stock solution was added. The tubes were mixed by inverting and centrifuged briefly. Samples were incubated at room temperature for 15 min.*
9. *320µl of Buffer RBC was added and mixed thoroughly by pipetting. We added 1120µl of ethanol (100%) and after mixing by pipetting, 700µl of sample was transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube. Samples were centrifuged for 15 s at 11,000 x g and the flow-through was discarded. This was repeated until the entire sample had passed through the spin column.*
10. *500µl of Buffer RPE was added to the spin columns and they were centrifuged for 15 s at 11,000 x g. The flow-through was discarded. Again 500µl of Buffer RPE was added, and the samples were centrifuged for 2 min at 11,000 x g, after which the collection tubes were discarded with the flow-through.*
11. *The spin columns were placed in new collection tubes. Tubes were centrifuged at full speed (16,100 x g) for 5 min with the lids of the columns open. Collection tubes were discarded with the flow-through.*
12. *Spin columns were placed into new microcentrifuge tubes, and 20µl of RNase-free water was added directly to the spin column membranes. Tubes were centrifuged for 1 min at full speed. The result was RNA elution of about 18µl per sample.*

The NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used for quantification of RNA. One MCV-negative tumor sample was excluded from the study because of insufficient RNA concentration. RNA was stored in a -70°C freezer until the Reverse transcription to cDNA was commenced.

2.3 Quantitative Reverse Transcription PCR

Reverse transcription of the RNA was done using the miScript II RT Kit (50) (QIAGEN cat.no. 218161). Each tumor RNA sample was diluted to a concentration of 300ng/µl, and 1µl of dilution was added to the Reverse transcription reaction components, which included: 4µl miScript HiSpec Buffer, 2µl miScript Nucleics Mix, 11µl RNase-free water. The control RNA sample was diluted to 500ng/µl, and 1µl of the dilution was added to reverse transcription reaction components.

Samples were first incubated for 60 min at 37°C, then for 5 min at 95°C to inactivate the reverse transcriptase mix. Afterwards the samples were placed on ice, and 5µl of each sample was transferred to new tubes and mixed with 10µl of RNase-free water. This resulted with cDNA aliquots with a concentration of 5ng/µl. In addition, the cDNA derived from the control skin sample was diluted with RNase-free water to produce an aliquot with concentration of 5ng/µl. The cDNA was stored in a -20°C freezer until the PCR was started.

Quantitative RT-PCR was executed with miScript SYBR Green PCR Kit (QIAGEN cat.no. 2180739) and LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany). 5ng of cDNA was mixed in PCR plate with the reaction components, including 10µl SYBR Green PCR mix, 2µl Universal Primer, 2µl primer for the miRNA desired to be amplified and 5µl RNase-free water. Each sample was transferred to a LightCycler capillary and placed into the LightCycler. PCR cycling conditions consisted of an initial activation step at 95°C for 15 minutes followed by 50 cycles with denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 70°C for 30 seconds.

PCR was performed on all of the samples with 6 different primers. The primers for the amplification of miRNAs: mir-34a, mir-30a, mir-181d, miR-142-3p, mir-1539 and the small nuclear RNA (snRNA) U6 were purchased from QIAGEN. U6 expression was used as a reference in statistical analysis. U6 is well suited for this purpose due to its high conservation throughout the evolution of many species.

Every PCR run consisted of a certain number of tumor samples as well as the skin control sample, which acted as a calibrator of the miRNA expression in statistical analysis, and negative control sample, RNase-free water, which was used to observe possible contamination. Each tumor sample and the control sample had a replica on the PCR plate to increase the accuracy of the experiment. Melting curve analysis was also performed to check for nonspecific amplification.

2.4 Statistical Analysis

Ct values (threshold cycle) for every sample and its replica were collected to a Microsoft Office Excel file from the LightCycler software, and the mean Ct value was calculated for each sample. We used the ΔΔCt method for the relative quantification of miRNA expression. The equation used: $\Delta\Delta Ct = (Ct_{miRNA} - Ct_{U6})_{control\ skin} - (Ct_{miRNA} - Ct_{U6})_{tumor\ sample}$

The relative quantity (RQ) for each miRNA, compared with the quantity of U6 was calculated using equation $2^{-\Delta\Delta Ct}$. Mean and median RQs for MCV-positive and MCV-negative tumors were calculated for every miRNA. The student's t-test was used to evaluate statistically significant differences in miRNA expression between the two groups: the MCV-negative tumors and MCV-positive tumors. MiRNA expression according to MCV-status was confirmed with the Mann-Whitney U-test using the median RQs. In addition to comparison according to MCV-status, tumors were divided into subgroups based on metastasis and location on sun exposed versus non-exposed areas (Figure 1.). The Mann-Whitney U-test was used to compare miRNA expression in these groups (location, metastasis). P-values of less than 0.05 were considered statistically significant.

3 Results

This study comprised 26 MCC patients, with 18 (69%) females and a mean age of 78. The mean tumor size was 3cm, ranging from 1cm to 8.5cm. Tumors were most commonly located in the head and neck 12 (46%) or limbs, and only five (20%) tumors were located in the trunk.

12 patients (46%) had previous chronic diseases, including cancers, for example breast or prostate cancer, and coronary disease. Three patients had immunocompromising diseases such as psoriasis and diabetes, and one patient was immunosuppressed following a kidney transplant. Nine (35%) patients developed local or distant metastasis during the course of disease. Six (23%) patients (patients number 2, 7, 8, 9, 11 and 17) were alive five years after the MCC diagnosis.

14 of the tumors were MCV-positive and 12 MCV-negative. 9 (75%) of the MCV-negative tumors were located in the head and neck area or limbs. Tumor locations are illustrated in Figure 1, and detailed clinical data are presented in Table 3.

The male patients' tumors had mean RQs of 1.49 for miR-34a, 2.30 for miR-30a, 1.61 for miR-142-3p, 0.67 for miR-181d and 5.46 for miR-1539. For the females, the corresponding values were 0.85, 2.14, 1.27, 0.30 and 1.30. Patients under 78 years had mean RQs of 1.03, 2.14, 1.80, 0.57 and 5.16. For the patients 78-years-old or older, the values were 1.06, 2.22, 1.15, 0.33 and 1.21.

MiR-34a, miR-30a, miR-1539, miR-142-3p were downregulated in MCV negative tumors compared with MCV positive tumors. Yet, only mir-34a expression reached statistical significance ($p=0.0063$). MiR-181d was slightly up- or downregulated depending on which were used in comparison, the median or mean RQ values. Detailed results of the PCR are presented in Tables 1 and 2. There was no statistical significant difference in miRNA expression according to tumor location or metastasis.

Table 1.

	MCV positive[1]	MCV negative[1]	p-value[2]	expression(neg. vs pos.)
miR-34a	1.53	0.49	0.0063	underexpressed
miR-30a	2.39	1.96	0.41	underexpressed
miR-1539	3.53	1.47	0.32	underexpressed
miR-142-3p	1.89	0.77	0.081	underexpressed
miR-181d	0.42	0.40	0.91	underexpressed

[1] Mean Relative quantity (RQ) values calculated using the equation $2^{-\Delta\Delta Ct}$.
[2] Calculated using Student's t-test

Table 2.

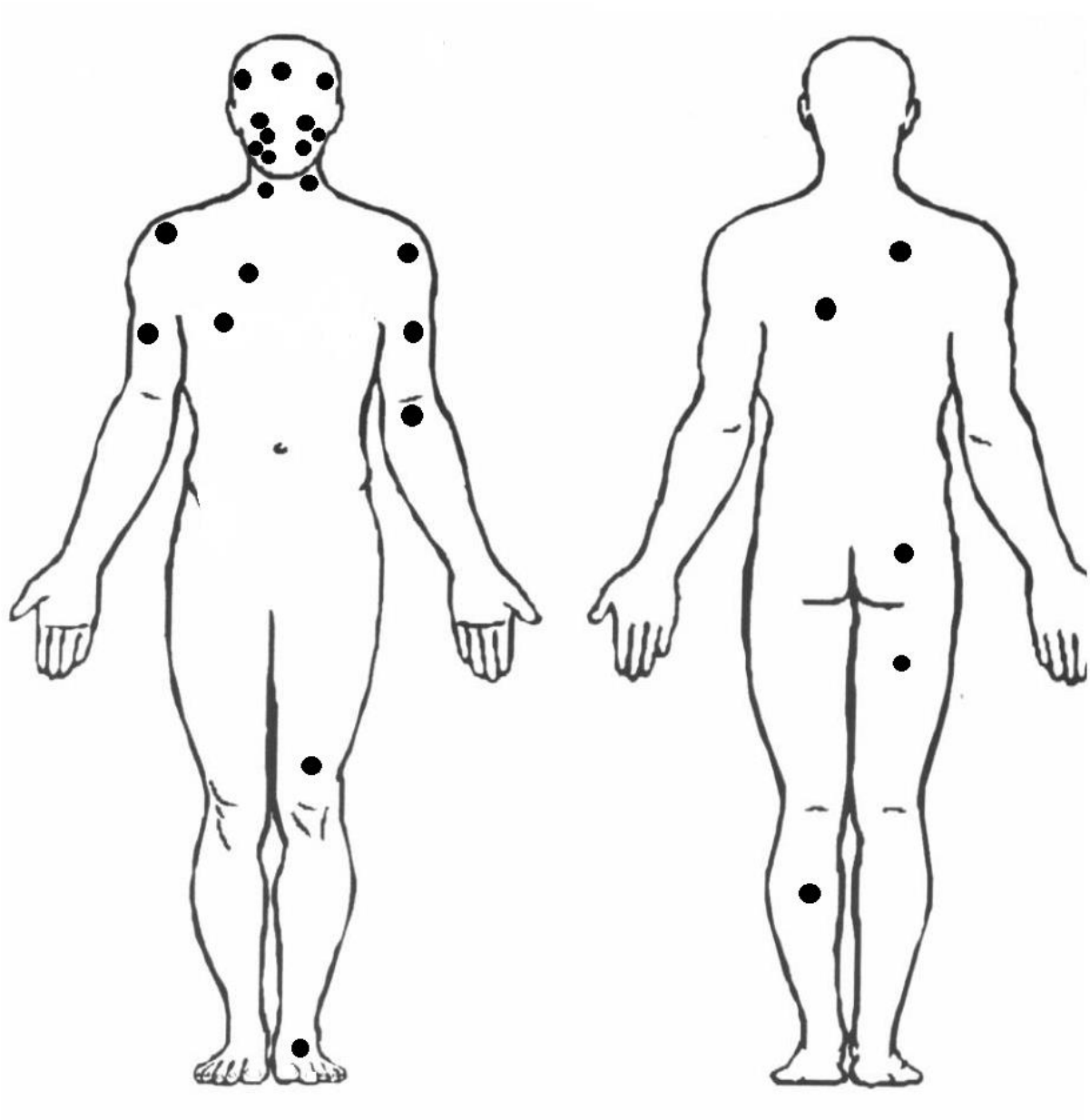
	MCV positive[3]	MCV negative[3]	p-value[4]	expression(neg. vs pos.)
miR-34a	1.16	0.23	0.0043	underexpressed
miR-30a	1.80	1.56	0.49	underexpressed
miR-1539	1.17	0.66	0.17	underexpressed
miR-142-3p	1.29	0.56	0.11	underexpressed
miR-181d	0.26	0.38	1.00	overexpressed

[3] Median Relative quantity (RQ) values using the equation $2^{-\Delta\Delta Ct}$.
[4] Calculated using Mann-Whitney U-test

Table 3.

Patient	Sex	Age at diagnosis	MCV status	Tumor size (mm)	Tumor location	Preceding Diseases	Metastasis location
1	F	90	negative	20	Right Temple		Right neck/chin
2	M	68	positive		Right cheek	Coronary disease	Scalp
3	F	80	positive	85	Back of the thigh		axillar and inguinal lymph nodes
4	M	59	positive	70	Chest		
5	M	67	negative	15	Left cheek	Kidney transplant	
6	F	72	positive	12	left knee	Parkinson's disease	
7	F	81	positive	20	Right cheek		
8	F	83	negative	50	Right arm		
9	F	85	negative	15	Left temple		
10	F	91	positive	30	forehead		
11	M	71	positive	34	Right buttock		
12	F	95	positive	18	left cheek		
13	F	87	positive	30	Left shoulder	Coronary disease	axillar lymph node, right thigh
14	F	77	negative	20	Right cheek	Diabetes. Coronary disease	Mediastinum. pleura and frontal lobe
15	F	79	negative	20	Right breast	Breast cancer	
16	F	72	negative	13	Calf		inguinal lymph nodes
17	F	57	positive	33	Right cheek		
18	M	78	negative	25	Neck	Psoriasis. solar keratosis	Anal canal. pancreas
19	M	79	positive	40	Left forearm		
20	F	81	negative		Left upper back	Coronary disease. osteoporosis	
21	F	84	positive	24	Right shoulder	Diabetes	
22	M	82	negative	28	Neck	Prostate cancer. solar keratosis	
23	M	85	positive	75	Left arm	non-Hodking lymphoma	axillar lymph nodes
24	F	84	negative	15	Back	Breast cancer	
25	F	87	positive	20	Cheek		
26	F	60	negative	10	Left arm		Heart. lung

Figure 1.



Tumors in the head and neck region and the tumor in the left forearm were considered to be located in a sun-exposed area.

4 Discussion

In this study, the expression profiles of five microRNAs (miR-34a, miR-1539, miR-30a, miR-142-3p and miR-181d) were compared according to the MCV status of the MCC tumors using quantitative RT-PCR. In consensus with our previously obtained microarray results, we confirmed statistically significant underexpression of miR-34a in MCV-negative tumor samples compared with MCV-positive tumors (p=0.0063).

MiR-34a overexpression in MCV-positive versus MCV-negative MCC was first reported by Xie et al (2013) (56). Similar to us, they first used microarray analysis to define global miRNA profiles of MCC tumors according to MCV-status and then used qRT-PCR for validation of the most significant expression differences. In both studies, microarray analysis yielded miR-34a and miR-30a overexpression in MCV-positive tumors. Otherwise, the miRNAs selected for the qRT-PCR validation in this current study were different; Xie et al. confirmed significant overexpression of miR-375, miR-769-5p and underexpression of miR-203 in MCV-positive tumors (56). Based on these findings, it is reasonable to propose that there might be a deviation in miRNA profiles regardless of MCV-status. However, since both of these independent studies found miR-34a to be differently expressed according to MCV status, it is possible that there are few key miRNAs whose dysregulation is consequential in MCC. In this study, the mean miR-142-3p relative quantity was over 2.5-times higher in MCV-positive tumors compared with MCV-negatives, and this finding almost reached a statistical significance.

Rodig et al. 2012 stated that all MCC tumors could actually contain MCV DNA (58). They used antibodies for the MCV Large T antigen to detect LTA in 97% of the tumor samples tested and also found MCV DNA in every 60 tumors tested with PCR analysis using a broadened set of MCV DNA specific primers. However, differences in miRNA expression suggest that there in fact are MCV-negative tumors whose pathophysiology may deviate to some extent from that of MCV-positives'. DNA methylation, Histone modification and mutations modify miRNA expression, and MCV viral proteins could potentially interfere with these modifications. Furthermore, dysregulation of miRNAs can lead to alterations in cell proliferation or initiation of apoptosis through abnormal gene expression.

MiR-34a has p53 dependent tumor suppressor properties (59,60). It forms a positive feedback loop with p53 as the activation of p53 by miR-34a increases the production of miR-34a. This leads to further suppression of miR-34a target genes, one of which is the SIRT1 gene. SIRT1, an enzyme of the sirtuin protein family, deacetylates p53 abolishing the tumor suppressive effects of p53. MiR-34a tumor suppressor properties, therefore, are at least partly explained by blocking SIRT1 (60,61). Sihto et al. reported that in MCC p53 expression is associated with *TP53 gene* mutations, which are observed by immunohistochemistry only in MCV LTA negative MCC tumors. This suggests that p53 could be involved in the tumorigenesis of MCV-negative tumors (62). They found p53 expression infrequently in MCV LTA positive tumors, and lower copy numbers of the MCV DNA were observed in p53-positive tumors compared with p53-negatives (62). The underexpression of miR-34a in MCV-negative tumors might have oncogenic effects.

Another transcription factor that regulates miR-34a is c-Myc. Unlike p53, c-Myc suppresses miR-34a expression (63). Sotillo et al. (2011) reported that miR-34a enhances cell survival by reducing p53 levels in c-Myc overexpressed Burkitt's lymphoma (BL) cell lines in the presence of chemotherapy (64). They suggested that miR-34a could have implications for other tumors with c-Myc deregulation and that miR-34a could be considered as a therapeutical target in such malignancies (64). One study reported that 70% of the tested MCC samples expressed c-Myc (65). Although c-Myc expression was not correlated with MCV-status (65), miR-34a could have a connection with c-Myc also in MCC, if even a slight miR-34a expression could interfere with c-Myc. Further, in MCV-positive tumors, miR-34a could lower p53 expression via c-Myc more efficiently due to overexpression of miR-34a. Moreover, this could potentially promote drug resistance as in BL cell lines (64).

Sahi et al. (2012) reported that the expression of anti-apoptotic protein bcl-2 indicates better prognosis of MCC patients (66). Mir-34a is a negative regulator of bcl-2 (67), and overexpression of miR-34a has been reported to inhibit bcl-2 in hepatocellular carcinoma (68). Since bcl-2 expression is a positive prognostic factor in MCC, the function of miR-34a in MCC might not be tumor suppressive if its overexpression inhibits bcl-2. This also suggests that the MCV-tumor positive patients reported preferable course of the disease could not be explained solely with miR-34a overexpression (39).

T lymphocyte infiltration within the tumor tissue and strong T cell infiltration indicates a better prognosis in MCC (69,70). Interestingly, Shin et al. reported that miR-34a increases T cell activation (71). Could there be a connection between MCV-positive tumors, overexpression of miR-34a and preferable course of the disease due to enhanced activation of T cells? However, this would require that miR-34a was also overexpressed in the infiltrating T cells.

Prominent miR-34a reduction in HPV induced cervical cancer was reported by Wang et al. They showed that a viral oncoprotein E6 reduces miR-34a expression by destabilizing p53, which enables cells to proliferate (72). miR-34a was also suggested to have unknown p53-independent activation mechanisms (72). In contrast, we showed that MCC tumors with MCV infection are associated with greater expression of miR-34a than MCV negative tumors. This inspires one to ask, does MCV infection affect miR-34a expression in MCC and if its action is to upregulate miR-34a, does the dysregulation of this miRNA impact the progression of this cancer.

Patients whose tumors were included in the study presented with several features typical for MCC. The age distribution was normal for MCC and the majority (80%) of the tumors were located in the head and neck region or limbs. In the highest UV-radiation exposed area, the head and neck region, there were 12 tumors (46%), which reminds that UV-radiation has an eminent causation to MCC. On the contrary, the five-year survival rate in this study was much lower than the rate usually reported (25-28). This could be partly explained by previous diseases that 46% of the patients had, but also by the fact that nine patients' cancers progressed to a metastasized state. We did not find a correlation between miRNA expressions and location of the tumor or progression of metastasis. Nevertheless, it would not be overly surprising should further studies show such correlations. Quite interesting was that mir34a RQ value was 5- to 19-times greater in tumor from the only patient who progressed to metastatic disease compared with other six tumors taken from those patients that survived more than 5 years.

The limitations of this study are the small sample size and scarce tumor material; then again there are not much more extensive tumor series anywhere due to the rareness of this cancer. Strengths of the present study are that the qRT-PCR yielded results

consistent with the microarray analysis and that the statistical analysis was done with two different methods, the student's t-test and the Mann-Whitney U-test.

Possible future directions would be to compare the expression of bcl-2 and miRNAs, especially miR-34a, in MCC tumors and to further compare the expression of miRNAs in primary tumors and their metastases and also the adjacent non-tumorous skin in order to find out the extent of miRNA dysregulation. An additional approach would be examine MCC tumors for T cell infiltration and compare to miRNA expression and survival of patients.

In conclusion, we found significant underexpression of miR-34a in MCV-negative tumors. In addition, miR-30a, miR-1539 and miR-142-3p were underexpressed in MCV-negative tumors compared to MCV-positives, but these findings failed to reach statistical significance. These results suggest that MCV-positive tumorigenesis potentially diverges from the MCV-negatives'. Essentially the underexpression of miR-34a might be consequential in the tumorigenesis of MCV-negative tumors.

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